

Contribution of MT1-MMP and of human laminin-5 $\gamma 2$ chain degradation to mammary epithelial cell migration

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SUMMARY

Membrane-type matrix metalloproteinase 1 (MT1-MMP) is a membrane-anchored matrix metalloproteinase (MMP) that is frequently associated with processes involving tissue remodelling and cell migration. We have examined MT1-MMP expression and subcellular distribution as a function of MCF10A mammary epithelial cell migration using an in vitro outgrowth migration assay. Stronger expression of MT1-MMP was observed at the mRNA and at the protein level in cells at the periphery of the outgrowth. As shown by videomicroscopy, these cells were involved in an orientated cell migration, in contrast to stationary cells distant from the periphery. Furthermore, MT1-MMP was mainly distributed in lamellipodia of migratory cells, as well as at their basal surface in contact with the substrate. Laminin-5 (Ln-5), a recently described substrate for MT1-MMP, was deposited preferentially in the matrix by migratory cells. Fragments of the $\gamma 2$ subunit of Ln-5 were also identified in migratory cultures of MCF10A cells, attesting to its proteolytic degradation. These fragments corresponded in size to those we observed after incubation of purified human Ln-5 with the recombinant catalytic domain of human MT1-MMP. We also show that anti-Ln5 blocking antibodies, MMP inhibitors (BB94 and TIMP-2) and MT1-MMP antisense oligonucleotides significantly decreased MCF10A cell migration. Taken together, these observations demonstrate that MT1-MMP is spatially and temporally regulated during MCF10A cell migration, and suggest that MT1-MMP-mediated pericellular proteolysis of Ln-5 $\gamma 2$ chain could contribute to this process.

Key words: MT1-MMP, Laminin-5, Migration, MCF10A, Epithelial cells

INTRODUCTION

Epithelial cell migration plays a central role in several physiological or pathological processes, including wound healing, organogenesis, placentation or tumour invasion. The acquisition of a migratory phenotype by epithelial cells is a spatially and temporally regulated process that involves integrated modifications of the cells and of their surrounding environment.

The remodelling of the extracellular matrix (ECM) surrounding epithelial cells is accordingly a mechanism frequently associated with cell migration. Matrix metalloproteases (MMPs), a family of proteases that degrades specific components of the ECM, have largely been implicated in the ECM degradation associated with many processes that involve epithelial cell migration (Matrisian, 1992; Werb, 1997; Shapiro, 1998; Murphy and Gavrilovic, 1999; Nagase and Woessner, 1999). Most MMPs are secreted as inactive proenzymes and their activation requires the proteolytic removal of the N-terminal pro-fragment (Nagase and Woessner, 1999). By contrast, MT1-MMP (membrane type-MMP) contains a transmembrane domain, which ensures the anchorage of the protein at the cell membrane (Sato et al., 1994; Sato and Seiki, 1996). MT1-MMP has been described as a major activator of MMP-2 (Sato et al., 1994; Sato and Seiki, 1996) but MT1-MMP also possesses the ability to degrade ECM components, including gelatin, K-elastin, fibronectin, vitronectin, native fibrillar type I, II and III collagen, and laminin-5 (Ln-5; Imai et al., 1996; Pei and Weiss, 1996; Sato and Seiki, 1996; Ohuchi et al., 1997; Koshikawa et al., 2000). The activity of MMPs, including MT1-MMP, is regulated by specific tissue inhibitors of MMPs (TIMPs). MMP-2 is unique in its binding of TIMP-2, and a trimolecular complex formed by MT1-MMP, TIMP-2 and MMP-2 has been described (Strongin et al., 1995). The expression of MT1-MMP has been detected in processes that involve epithelial cell migration and ECM remodelling, and has been correlated to high invasive abilities in tumour cell lines (Gilles et al., 1996; Sato and Seiki, 1996; Gilles et al., 1997; Pulyaeva

et al., 1997; Polette et al., 1998; Quaranta, 2000). However, the mechanisms by which MMPs and particularly MT1-MMP facilitate migration are still poorly understood. The anchoring of MT1-MMP at the plasma membrane is thought to contribute to a pericellular ECM degradation, either by its own degradative potential or through its ability to activate MMP-2 at the cell surface (Nakahara et al., 1997; Chen and Wang, 1999; Quaranta, 2000). A crucial issue is therefore the determination of the expression and spatial distribution of MT1-MMP in epithelial cells during cell migration. Indeed, a pericellular degradation now appears as a key event, generating an adequate substrate for cell migration in the immediate vicinity of migrating cells (Nakahara et al., 1997; Chen and Wang, 1999; Murphy and Gavrilovic, 1999; Hotary et al., 2000; Quaranta, 2000).

Accordingly, rat laminin-5 (Ln-5) has recently been identified as a substrate for both MT1-MMP and MMP-2 (Giannelli et al., 1997; Koshikawa et al., 2000). Ln-5 is a heterotrimer found in basement membranes (BM), which consists in the association of $\alpha 3$, $\beta 3$ and $\gamma 2$ subunits (Rousselle et al., 1991). It is a multifunctional protein that, in apparent contrast, is involved in the static adhesion of epithelial cells to the basement membrane (BM; Baker et al., 1996; Jones et al., 1998; Borradori and Sonnenberg, 1999; Nievers et al., 1999) and also in the promotion of epithelial cell migration (Miyazaki et al., 1993; Zhang and Kramer, 1996; Giannelli et al., 1997; Grassi et al., 1999; Goldfinger et al., 1999; Koshikawa et al., 2000). The proteolytic degradation of Ln-5 $\alpha 3$ and $\gamma 2$ subunits generates different heterotrimeric forms of Ln-5 (Rousselle et al., 1991; Marinkovich et al., 1992; Vailly et al., 1994; Matsui et al., 1995), and appears to be responsible for the contrasting activities (i.e. adhesion or migration) attributed to Ln-5 (Giannelli et al., 1997; Goldfinger et al., 1998; Giannelli et al., 1999; Goldfinger et al., 1999; Koshikawa et al., 2000). Recent data have shown that the degradation of $\gamma 2$ subunit of exogenously provided rat Ln-5 by MMP-2 and/or MT1-MMP promotes cell migration in vitro (Giannelli et al., 1997; Giannelli et al., 1999; Koshikawa et al., 2000). Furthermore, evidence that the degradation of Ln-5 $\gamma 2$ chain could promote cell migration in vivo has been given by the detection of specific fragments of $\gamma 2$ in remodelling but not in quiescent mouse tissues and in rodent skin carcinoma (Giannelli et al., 1997; Giannelli et al., 1999).

In this study, we have used an in vitro migration assay coupled to videomicroscopy analyses to study the expression and cellular distribution of MT1-MMP in association with cell migration of human epithelial mammary MCF10A cells. We show a stronger expression of MT1-MMP mRNA and protein in migratory cells, and a distribution of MT1-MMP in lamellipodia and at the basal surface of these migratory cells. Furthermore, we have found that Ln-5 was deposited around migratory cells and also detected degraded fragments of Ln-5 $\gamma 2$ subunit in migratory cultures of MCF10A cells. We have also used MMPs inhibitors (BB94 and TIMP-2), MT1-MMP antisense oligonucleotides and Ln-5 blocking antibodies, which emphasized a functional contribution of MT1-MMP and Ln-5 in cell migration.

MATERIALS AND METHODS

Cell culture

Human mammary epithelial MCF10A cells were obtained from the American Type Culture Collection (Rockville, MD). Their growth medium was composed of HAM F12 and Dulbecco's modified Eagle medium (DMEM) 1:3 (v/v) supplemented with 20 $\mu\text{g/ml}$ of adenine, 5 $\mu\text{g/ml}$ of insulin, 0.5 $\mu\text{g/ml}$ of hydrocortisone, 2 ng/ml of EGF, 5 $\mu\text{g/ml}$ of transferrin, 1.5 ng/ml of triiodothyronine and 10% foetal calf serum.

In vitro migration assay

5×10^4 cells were seeded in growth medium inside a 6 mm glass ring placed in the middle of a glass coverslip (22 mm in diameter). Twenty-four hours after plating, the glass ring was removed and the cells were covered with growth medium. In this model, the cells migrate as an outgrowth from the confluent area initially delimited by the ring.

For epidermal growth factor (EGF)-induced migration, cells were plated inside the ring in complete growth medium for 24 hours. After the removal of the ring, cells were washed for 1 hour twice in serum-free medium and then covered with serum-free medium supplemented, or not, with EGF at 20 ng/ml.

For TIMP-2 and BB94 inhibition experiments, 72 hours after the ring removal TIMP-2 (at 1 $\mu\text{g/ml}$, Calbiochem, La Jolla, CA) and BB-94 (5×10^{-6} M, kindly provided by British Biotech, Oxford, UK) were added to the growth medium for 1 hour, before the quantification of cell migration.

For MT1-MMP antisense experiments, a T1-MMP antisense oligonucleotides kit was used (Biognostik, Gottingen, Germany). The control oligonucleotides were scrambled oligonucleotides of the MT1-MMP antisense

oligonucleotides. The oligonucleotides were added in the growth medium at 4 μ M (as recommended by the manufacturer) after the removal of the ring. Using FITC-labelled oligonucleotides, we determined that 144 hours were necessary to ensure an uptake of the oligonucleotides by at least 20% of the cells. The effect of control and MT1-MMP antisense oligonucleotides on cell migration were therefore measured 6 days after the removal of the ring and the addition of the oligonucleotides.

For Ln-5 antibody inhibition experiments, mouse Ln-5 blocking antibodies (clone P3H9-2, Bioproducts, Heidelberg, Germany) were added to the culture medium (at 25 μ g/ml) 72 hours after the removal of the ring and incubated for 4 hours before quantification of the cell migration speed. Controls were incubated with mouse IgG.

Quantification of cell migration speed and cell trajectories

To quantify the expansion of the outgrowth, migratory monolayers were placed on the stage of an inverted microscope (Nikon TMS-F, Tokyo, Japan), connected to a video CCD camera (Cohu 4700, San Diego, CA) and a video monitor (PVM 1371, Sony, Japan), so as to measure the outgrowth area.

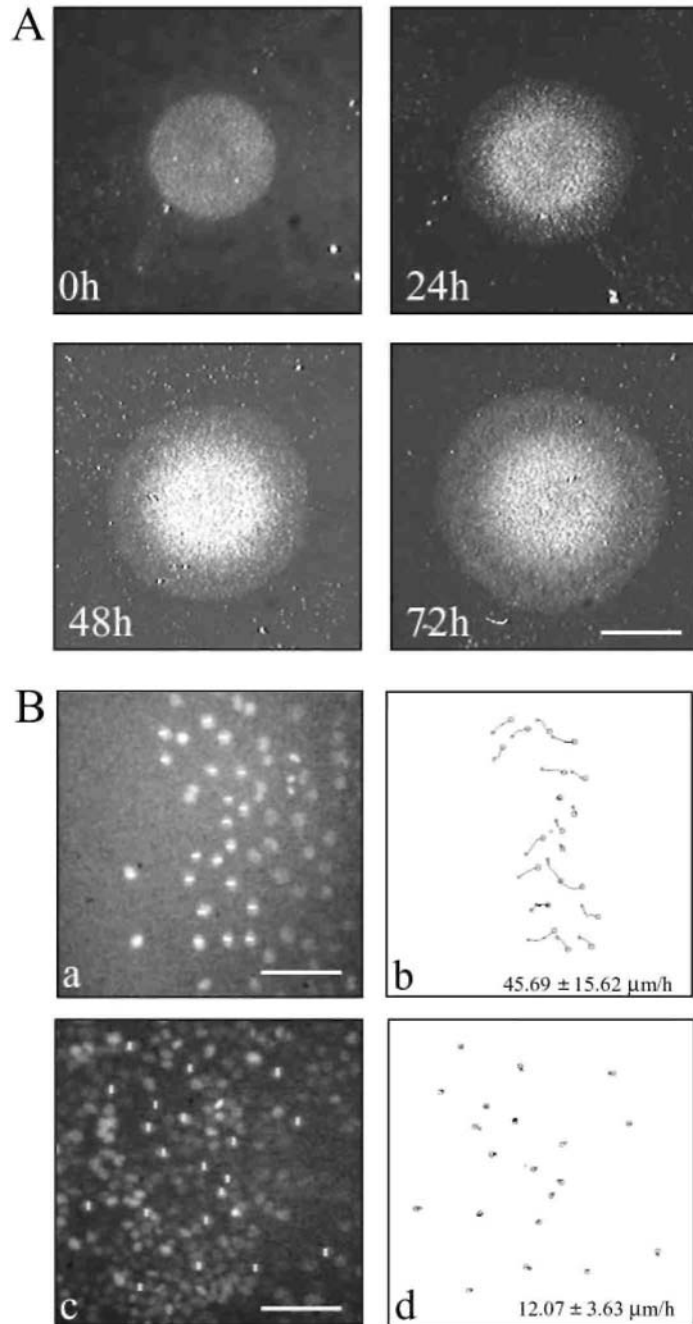
To analyse and quantify the migratory speeds and trajectories of the cells, the monolayers were first incubated with a fluorescent nuclear dye (Hoechst 33258, Molecular Probes, Eugene, OR). They were then placed in the environmental chamber (37°C, 5% CO₂) of a Zeiss IM35 inverted microscope (Zeiss, Oberkochen, Germany) equipped with an epifluorescence illumination source (excitation filter at 360 nm; emission filter at 510 nm) and a low level SIT camera (Lhesa 4036) controlled by a microcomputer (SparcClassic Workstation). An image was collected every 10 minutes for 30 minutes. Twenty cell nuclei selected in different zones of the culture were then labelled manually on the computer for each time point. Cell migration was characterized and quantified using a previously described software program (Zahm et al, 1997) that measures the nuclei trajectories, as well as the cell migration speed. As the cell nuclei were labelled manually by the experimenter, the relative position of the label on a nucleus pictured at different time points varied slightly. Consequently, a movement of the cells nuclei could be detected (corresponding to a speed of about 10 μ m/hour) in the areas distant from the outgrowth periphery. This movement was however random and was not considered as migration. Hence, the corresponding areas were identified as stationary. By contrast, the movement of the nuclei at the periphery of the outgrowth corresponding to cell migration was clearly oriented towards the outside of the outgrowth.

RT-PCR analyses

RNA extraction was performed from total migratory cultures using the RNA miniprep kits as recommended by the manufacturer (Qiagen, Hilden, Germany). RT-PCR was performed using 10 ng of total RNA. An internal control RNA template containing the sequences of the different primers used to amplify different MMPs was introduced in each sample for the standardization and quantification of each RT-PCR reaction.

RT-PCR was performed using the GeneAmp ThermoStable RNA PCR Kit (Perkin Elmer, Foster City, CA), and with pairs of primers for six MMPs and for 28S control amplification (Eurogentec, Seraing, Belgium). Forward and reverse primers for human MT1-MMP, MMP-2, MMP-9, MMP-1, MMP-3 and MMP-11 and 28S were designed as follows: MT1-MMP primers (forward 5'-CCATTGGGCATCCAGA-AGAGAGC-3'; reverse 5'-GGATACCCAATGCCATTGGCCA-3'), MMP-2 primers (forward 5'-GGCTGGTCAGTGGCTTGGGGTA-3'; reverse 5'-AGATCTTCTTCTCAAGGACCGGTT-3'), MMP-1 primers (forward 5'-GAGCAAACACATCTGAGGTACAGGA-3'; reverse 5'-TTGTCCCGATGATCTCCCCTGACA-3'), MMP-3 primers (forward 5'-GATCTCTTCATTTGGCCATCTCTTC-3'; reverse 5'-CTCCAGTATTTGTCCTCTACAAAGAA-3'), MMP-11 primers (forward 5'-ATTTGGTTCTTCCAAGGTGCTCAGT-3'; reverse 5'-CCTCGGAAGAAGTAGATCTTGTCT-3') and 28S primers (forward 5'-GTTCAACCACTAATAGGGAACGTGA-3'; reverse 5'-GGATTCTGACTTAGAGGCGTTCAGT-3'). Reverse transcription was performed at 70°C for 15 minutes. Amplification cycles were as follows: 15 seconds at 94°C, 15 seconds at 68°C, 10 seconds at 72°C. Twenty-five cycles were allowed for MT1-MMP amplification, up to 35 cycles for MMP-2, MMP-1, MMP-3 and MMP-11 amplification, and 18 cycles for 28S amplification. Products were separated on acrylamide gels, stained with Gelstar (FMC, Bioproducts) and quantified by fluorimetric scanning (LAS-1000, Fuji). The ratio of each endogenous signal to its specific internal control was calculated and normalized to its the ratio to the 28S. These values were multiplied by the number of copies of internal controls added to the RT-PCR reactions. Results were therefore expressed as a number of copies per 10 ng of RNA, allowing the comparison of the expression of different MMPs that have been amplified using different PCR parameters.

Fig. 1: *In vitro* migration assay. MCF10A cells were plated inside a glass ring and were allowed to migrate as an outgrowth after the ring removal. (A) Sequential video images of a migratory culture of MCF10A cells, taken immediately, 24, 48 or 72 hours after the removal of the ring and showing the expansion of the outgrowth. (B) Images of nuclei stained with Hoechst dye and visualized under epifluorescence illumination (a, at the edge of the outgrowth; c, distant from the outgrowth periphery). Trajectories of 20 randomly selected nuclei (indicated with a white dots in a and c) were quantified in each area (b, at the edge of the outgrowth; d, distant from the outgrowth periphery). Scale bars: 2.35 mm in A; 80 μ m in B.



Immunofluorescence

Monolayers were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at 37°C then treated with 0.1% Triton X-100 for MT1-MMP and integrin labelling. For Ln-5 labelling, the cells were fixed with methanol for 10 minutes at -20°C. The coverslips were then saturated for 30 minutes with 3% BSA in PBS.

For MT1-MMP immunostaining, monolayers were successively (after intermediate washes in PBS) incubated for 1 hour with a monoclonal antibody to MT1-MMP (clone 113-5B7 Chemicon, Temecula, CA), a biotinylated-sheep anti-mouse antibody (Amersham, Aylesbury, UK) and a Texas Red-conjugated streptavidin (Amersham).

For $\alpha 6$ integrin staining, cells were successively incubated with anti $\alpha 6$ integrin rat antibody (clone GoH3, Immunotech, Marseille, France), a biotinylated goat anti-rat antibody (Sigma) and a Texas Red-conjugated streptavidin (Amersham). For $\alpha 3$ integrin labelling, cells were incubated subsequently with an anti $\alpha 3$ monoclonal antibody (clone P1B5, Dako) and with a TRITC-conjugated rabbit anti-mouse antibody.

For Ln-5 labelling, the cells were successively incubated with an anti Ln-5 monoclonal antibody (clone GB3, described in Verrando et al., 1987), a biotinylated-sheep anti-mouse antibody (Amersham) and FITC-conjugated streptavidin (Amersham).

After incubation with the different antibodies, nuclei were labelled with 4',6-diamidino-2-phenylindole (DAPI; 1 $\mu\text{g/ml}$) for 20 minutes. The coverslips were then mounted with Aquapolymount antifading solution (Agar, UK) onto glass slides and the slides were observed under a Zeiss fluorescence microscope or with a MRC 600 confocal laser scanning microscope (BioRad, Richmond, CA).

In situ hybridization

The cultures were fixed for 10 minutes in 4% paraformaldehyde in PBS, dehydrated in ethanol 50% and 70%, rehydrated and treated with 0.2 N HCl for 20 minutes at room temperature. They were then washed in 2xSSC, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes and hybridized overnight with [^{35}S]-labelled MT1-MMP antisense RNA transcripts. This probe was prepared from the MT1-MMP cDNA insert that had been cloned into pBluescript. The samples were then treated with RNase (20 $\mu\text{g/ml}$) for 1 hour at 37°C to remove the unhybridized probes, washed under stringent conditions and detected autoradiographically by exposure to D19 emulsion (Kodak, Rochester, NY) for 15 days. The control slides were treated under the same conditions but were hybridized with [^{35}S]-labelled sense probes. Quantification of the in situ hybridizations was performed using the Discovery system automated image analyser (Becton-Dickinson, Mountain View, CA) that allowed the determination of the mean area of the in situ hybridization grains per cell. The mean area of the in situ hybridization grains per cell was measured automatically on 10 fields (500 cells) at high magnification (x500). In order to compare MT1-MMP mRNA expression in stationary versus migratory cells, we performed these measurements in the two rows of cells at the periphery of the outgrowth and in the following rows of the cells (between the fourth and the tenth rows of cells) on three independent experiments.

Degradation of purified human Ln-5 by recombinant MT1-MMP

Human Ln-5 was purified from human squamous carcinoma SCC25 cell conditioned medium as described previously (Rousselle et al., 1991). Purified Ln-5 (1 μg) was incubated with the recombinant catalytic domain of human MT1-MMP (100-500 ng, Chemicon) in 50 mM Tris pH 7.5, 5 mM CaCl_2 , 150 mM NaCl for 16 hours at 37°C. The samples were then analysed by western blotting for the expression of the $\gamma 2$ chain of Ln-5.

Western blotting analyses

Analyses of MT1-MMP and Ln-5 $\gamma 2$ chain expression were performed on protein extracts performed 72 hours after the removal of the ring on migratory cultures of MCF10A cells (cultivated in complete growth medium or in serum-free medium supplemented with EGF) or on stationary cultures of MCF10A cells (cultivated in EGF/FCS-free medium) for 72 hours after the removal of the ring.

Extracts were prepared by scraping the cells in RIPA buffer (50mM Tris (pH 7.4), 150 mM NaCl, 1% Igepal (v/v), 1% sodium deoxycholate (w/v), 5 mM iodoacetamide, 0.1% SDS (w/v)) containing protease inhibitors (1mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g/ml}$ leupeptin and 10 $\mu\text{g/ml}$ aprotinin).

Samples (8 μg for Ln-5 $\gamma 2$ chain analyses and 20 μg for MT1-MMP analyses) were mixed with 1/5 sample buffer (0.31 M Tris (pH6.8), 10% SDS (w/v), 25% glycerol (v/v), 12.5% β -mercaptoethanol (v/v) and 0.125% bromophenol blue (w/v)) and boiled for 5 minutes. They were then separated on 7.5% and 12% SDS-PAGE gels for Ln-5 and MT1-MMP analyses respectively and transferred to a PVDF filter (NEN, Boston, MA). The membranes were then blocked with 5% milk (w/v), 0.1% Tween 20 (w/v) in PBS for 2 hours before exposure to the primary antibody overnight at 4°C: a rabbit antibody (clone L γ 2-1) generated against the C-terminal region of Ln-5 $\gamma 2$ subunit or a monoclonal antibody directed against the hemopexin-like domain of MT1-MMP (clone

2D7 kindly provided by Dr Rio, IGBMC, Illkirch, France). The filters were then incubated either with a horseradish peroxidase-conjugated swine anti-rabbit or goat anti-mouse antibody (Dako). Signals were detected with an enhanced chemoluminescence (ECL) kit (NEN, Boston, MA).

Statistical analyses

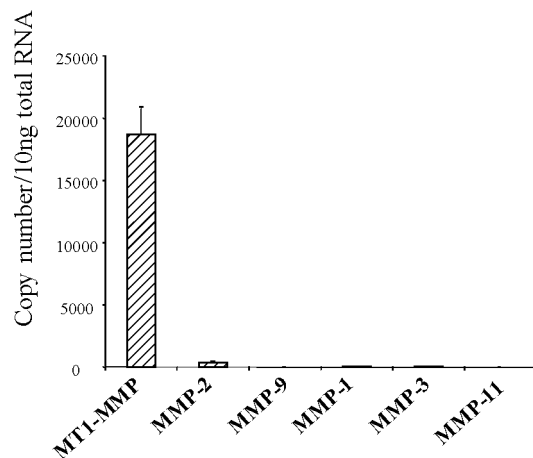
Experiments were performed in triplicate at least three times each. Data are expressed as means \pm s.d. Student's *t* test was used to compare the migration speeds of the cells under various experimental conditions. $P < 0.05$ was considered to be significant.

RESULTS

MT1-MMP is a major MMP expressed in migratory cultures of MCF10A cells

In order to examine the implication of MT1-MMP in epithelial cell migration, we used the human mammary MCF10A cells plated in an *in vitro* migration assay allowing the visualization and quantification of cell migration (Gilles et al., 1999). In this migration assay, cells are plated at a high density in a glass ring and migrate as an outgrowth after the removal of the ring (we will refer to these cultures as migratory cultures, Fig. 1A). Using videomicroscopy, we demonstrated that cells at the periphery of the outgrowth are involved in an orientated migration whereas cells distant from that periphery are basically stationary (Fig. 1B). Comparing the expression of MT1-MMP by quantitative RT-PCR with the expression of other MMPs, we found that MT1-MMP is a major MMP expressed in our model compared with other MMPs (MMP-9, MMP-2, MMP-1, MMP-3 and MMP-11), which were barely detectable (Fig. 2).

Fig. 2: Differential expression of MMPs mRNA in migratory cultures of MCF10A cells. RT-PCR analyses for MT1-MMP, MMP-2, MMP-9, MMP-1, MMP-3 and MMP-11 were performed on total RNA extracted from migratory cultures of MCF10A cells.

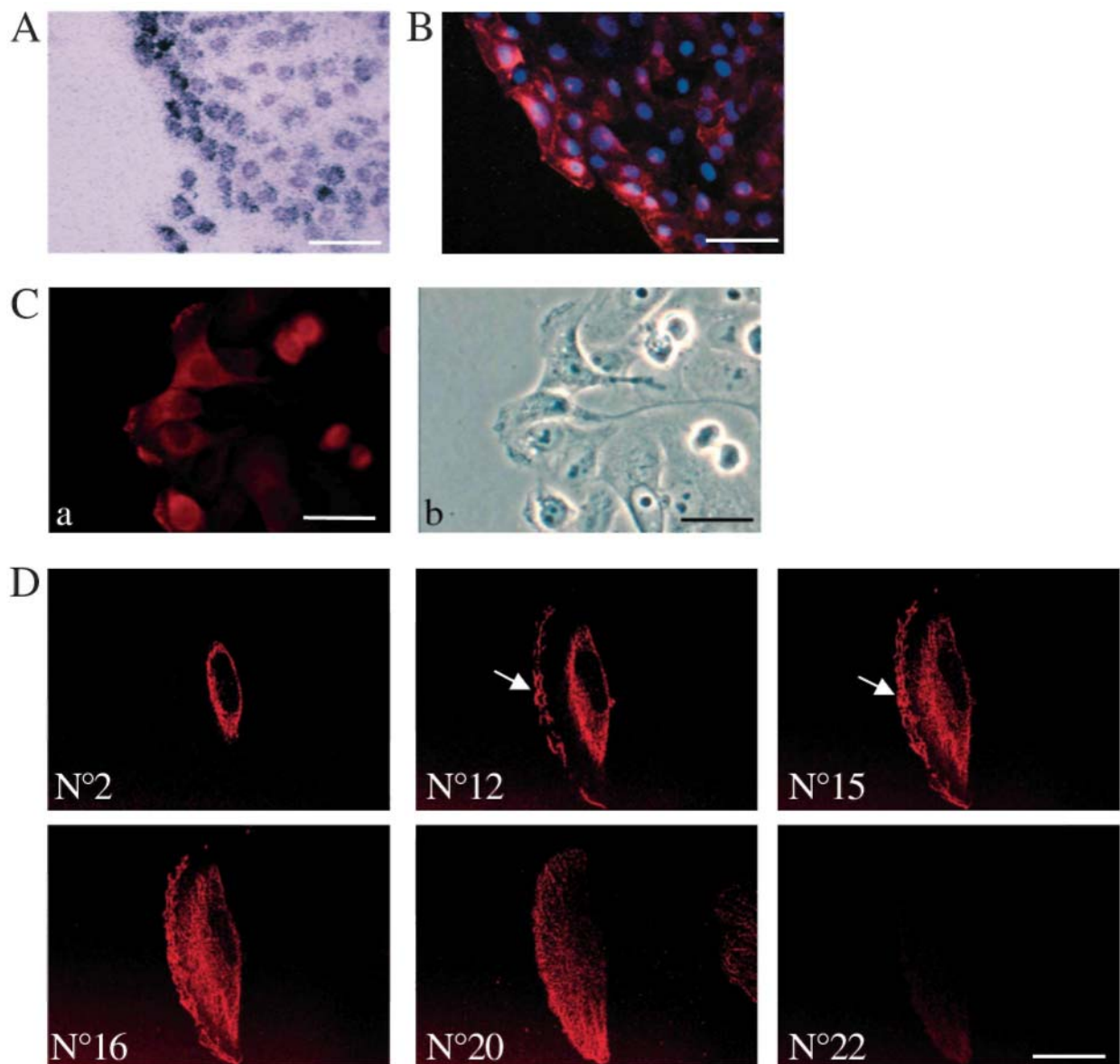


MT1-MMP is overexpressed in migratory MCF10A cell

In order to study more precisely the relationship between MT1-MMP and cell migration, we performed *in situ* hybridization and immunofluorescence analyses on migratory cultures of MCF10A cells. A stronger expression of MT1-MMP was clearly observed both at the mRNA (Fig. 3 A) and protein level (Fig. 3B) in cells at the periphery of the outgrowth, which has been shown to be a subpopulation of migratory cells. Densitometric quantification of the *in situ* hybridizations revealed a 2- to 2.4-fold increase in the density of grains in the rows of cells at the periphery of the outgrowth when compared to the density in the eight subsequent rows. Moreover, immunofluorescence combined with phase contrast analyses clearly revealed that MT1-MMP is present in lamellipodia identified as a phase-dark rim at the leading edge of the cells (Fig. 3C). Confocal microscopy confirmed this particular distribution of MT1-MMP in lamellipodia at the leading edge of migratory cells but also revealed its presence at the basal surface of these migratory cells in contact with the substrate (Fig. 3D).

Furthermore, using MMP inhibitors BB94 and TIMP-2, we showed a decrease of MCF10A cell migration (Fig. 4). The addition of MT1-MMP antisense oligonucleotides (with a maximal uptake of the oligonucleotides by about 20% of the cells) resulted in a decrease of MT1-MMP expression, as evaluated by immunofluorescence, and also diminished cell migration (Fig. 4). These data suggest a functional contribution of MT1-MMP overexpression and activity to MCF10A cell migration.

Fig. 3: Specific overexpression and subcellular organization of MT1-MMP in migratory MCF10A cells at the periphery of the outgrowth. (A) In situ hybridization on a migratory culture of MCF10A cells performed with a MT1-MMP antisense probe 72 hours after the removal of the ring. (B) MT1-MMP immunolabelling on a migratory culture of MCF10A cells. MT1-MMP labelling is in red and DAPI staining is in blue. (C) MT1-MMP immunolabelling (in red) on a migratory culture of MCF10A cells (a). Phase-contrast microscopy analysis of the corresponding area (b). (D) Confocal microscopy analyses of MT1-MMP in migratory MCF10A cells 72 hours after the removal of the ring. Twenty-four successive optical sections were taken from the apical (section 2) to the basal (section 22) surface of the cells (sections 2, 12, 15, 16, 20 and 22 are shown). MT1-MMP labelling was mainly found in the lamellipodia at the leading edge of migratory cells (arrows) as well as at the basal surface of the cells in contact with the substrate (section 16 and 20). Scale bars: 60 μ m in A,B; 20 μ m in C; 10 μ m in D.

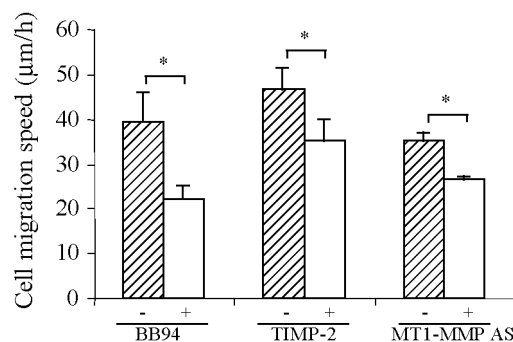


Ln-5 and MCF10A cell migration

AS rat Ln-5 has recently been described as a potential substrate for MT1-MMP (Koshikawa et al., 2000), the presence and deposition of endogenously produced human Ln-5 was investigated in migratory cultures of MCF10A cells. By immunofluorescence, Ln-5 appeared to be overexpressed by migratory cells at the periphery of the outgrowth (Fig. 5 A, part a). The deposition of Ln-5 was also emphasized by the presence of traces of Ln-5 behind cells which had migrated out of the outgrowth (Fig. 5A, part b). Ln-5 blocking antibodies were also found to decrease MCF10A cell migration suggesting a contribution of the interactions between MCF10A cells and Ln-5 in their migratory behaviour (Fig. 5B).

The distribution of $\alpha 6$ and $\alpha 3$ integrins, which constitute the $\alpha 6\beta 4$ and $\alpha 3\beta 1$ receptors known as two major receptors for Ln-5, was also examined by immunofluorescence as a function of MCF10A cell migration. These integrins were not only present in MCF10A migratory cultures but also reorganized in function of cell migration. They were indeed mainly found in lamellipodia of migratory cells, whereas in stationary cells, $\alpha 3$ was detected rather in places cell-cell contact and $\alpha 6$ at the basal surface (Fig. 5C). These results show that Ln-5 and its major receptors are expressed in our model but also display a specific distribution during cell migration.

Fig. 4: Inhibitory effect of BB94, TIMP-2 and MT1-MMP antisense oligonucleotides on MCF10A cell migration. 72 hours after the removal of the ring, BB-94 (at 5×10^{-6} M) and TIMP-2 (1 μ g/ml) were added for 1 hour before the quantification of migration speed of cells at the periphery of the outgrowth. MT1-MMP oligonucleotides antisense (+) or scrambled control oligonucleotides (-) were added to the medium immediately after the ring removal for 144 hours before the quantification of the cell migration speed at the periphery of the outgrowth. * $P < 0.05$.



Ln-5 degradation and MCF10A cell migration

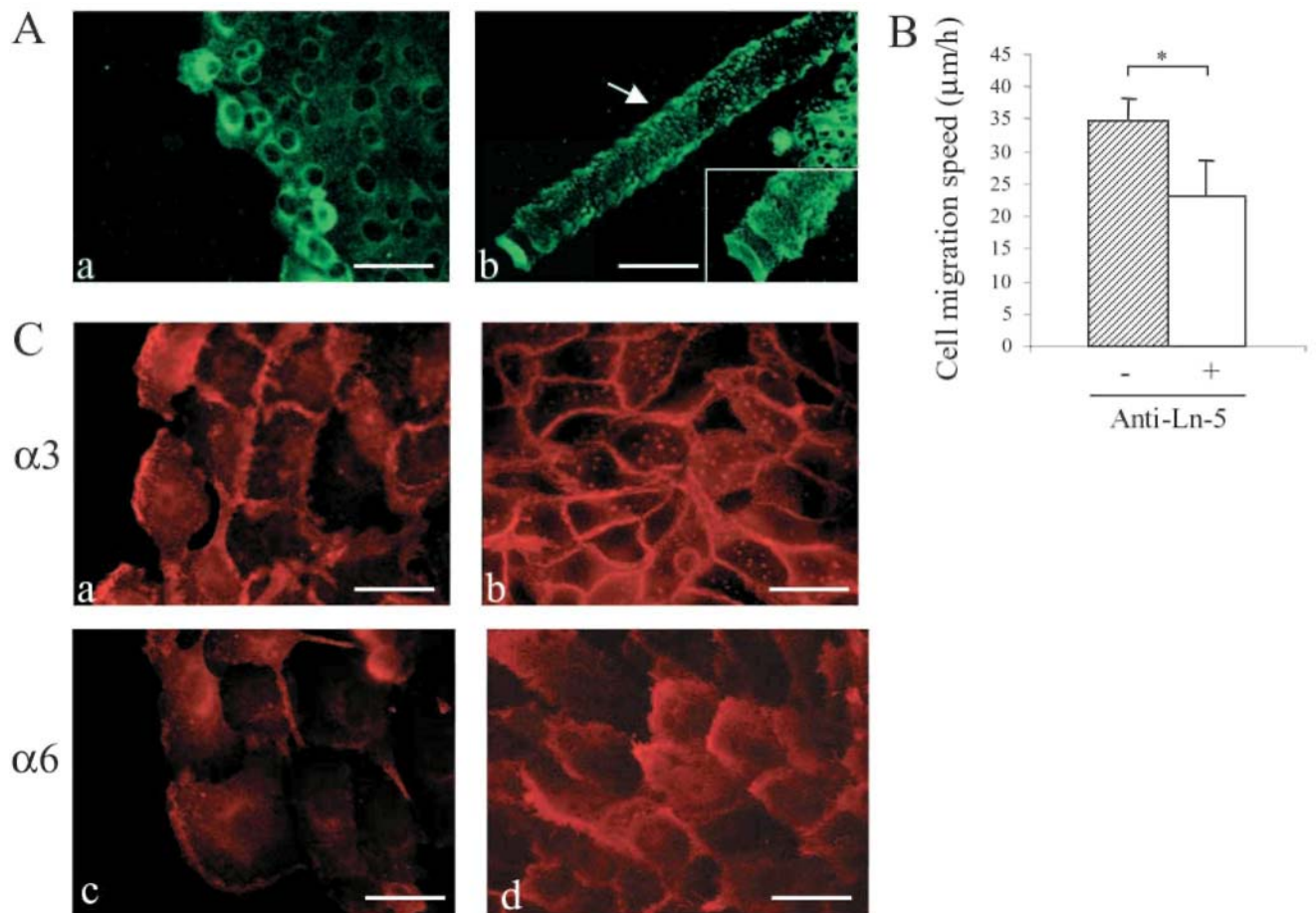
In order to examine the potential contribution of MT1-MMP on the degradation of Ln-5 $\gamma 2$ chain degradation during epithelial cell migration, the pattern of endogenously produced $\gamma 2$ chain of Ln-5 was examined by western blotting analyses in migratory cultures of MCF10A cells. Four Ln-5 $\gamma 2$ fragments could clearly be identified in total extracts of migratory MCF10A cultures (Fig. 6A). The two largest fragments corresponded in size to those detected in Ln-5 purified from squamous carcinoma SCC25 cell-conditioned medium, and have previously been described as the unprocessed human Ln-5 $\gamma 2$ subunit (155 kDa) and a processed fragment of human $\gamma 2$ (105 kDa; Rousselle et al., 1991).

Furthermore, the incubation of purified Ln-5 with the recombinant catalytic domain of human MT1-MMP generated fragments corresponding in size to those identified in our migratory cultures of MCF10A cells (Fig. 6A).

In order to relate the presence of the degraded fragments of Ln-5 $\gamma 2$ chain to cell migration, we compared the Ln-5 $\gamma 2$ pattern in migratory cultures (incubated in complete medium) with the one obtained in cells from cultures incubated in EGF/serum-free medium previously characterized as stationary cultures (Gilles et al., 1999). In order to minimize the potential contribution of serum-derived proteases in the processing of Ln-5 $\gamma 2$ chain, we also examined the $\gamma 2$ chain pattern in cells cultivated in serum-free medium supplemented with EGF, which has previously been shown to promote cell migration in our model (Gilles et al., 1999). It was verified

that, as observed for migratory cultures of MCF10A cells incubated in complete medium, both MT1-MMP and Ln-5 expression was increased specifically in cells at the periphery of the outgrowth in EGF-induced migratory cultures (data not shown). Using these different culture conditions, we could show increased amounts of Ln-5 $\gamma 2$ and its three degraded fragments in migratory cultures (incubated in serum- or in EGF-containing medium) in comparison with those detected in EGF/serum-free stationary cultures (Fig. 6B). As shown by MT1-MMP western blotting analyses, this increase in Ln-5 fragments in migratory cultures of MCF10A cells clearly correlated with increased amount of MT1-MMP (Fig. 6C). These results also revealed that MT1-MMP was mostly present as the 60 kDa form, known to be the active form of the enzyme. As MMP-2 has also been shown to cleave rat Ln-5 $\gamma 2$, we looked at MMP-2 expression by zymography analyses and did not find any detectable levels of MMP-2 in cell extracts of MCF10A cells cultivated with or without EGF in the migration assay (data not shown). These data therefore suggest that MT1-MMP could contribute to the degradation of endogenously produced Ln-5 $\gamma 2$ during cell migration.

Fig. 5: Ln-5 deposition in migratory cultures and contribution to MCF10A cell migration. (A) Ln-5 immunolabelling of migratory cultures of MCF10A cells 72 hours after the removal of the ring. Ln-5 was detected mainly in migratory cells at the periphery of the wound (a) but was also found deposited in the matrix as clearly shown by traces left behind cells that have migrated out of the outgrowth (b, arrow). (B) Seventy-two hours after the removal of the ring, Ln-5 blocking antibodies (clone P3H9-2) were added for 4 hours before the quantification of cell migration speed at the periphery of the outgrowth. * $P < 0.05$. (C) $\alpha 3$ and $\alpha 6$ integrins immunolabelling of migratory cultures of MCF10A cells. (a,c, periphery of the outgrowth; b,d, distant from the periphery of the outgrowth). Scale bars: 60 μm in Aa; 80 μm in Ab; 20 μm in C.



DISCUSSION

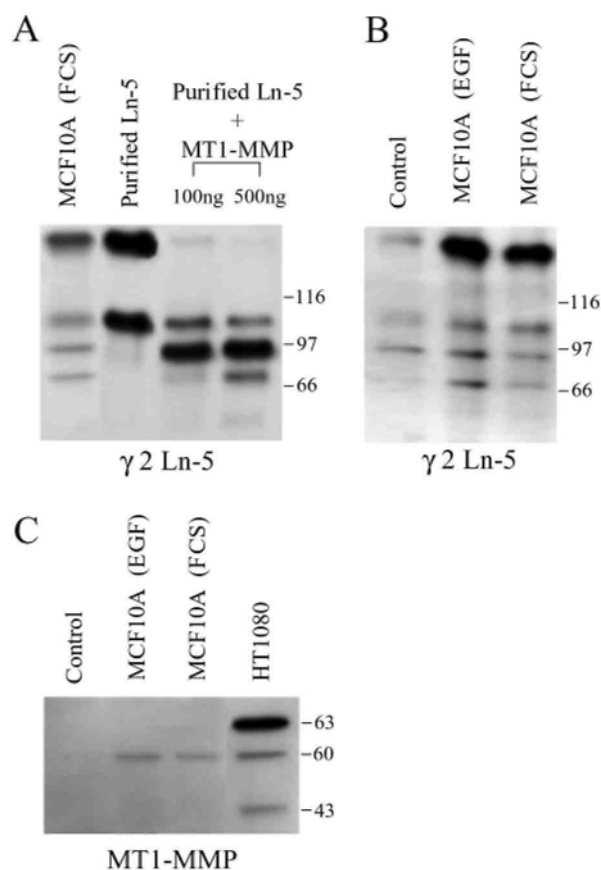
In the present study, we have shown that MT1-MMP is overexpressed and redistributed during human epithelial mammary MCF10A cell migration in association with Ln-5 $\gamma 2$ chain degradation. We indeed observed: (1) a stronger expression of MT1-MMP in migratory MCF10A cells versus stationary ones, by in situ hybridization, immunofluorescence and western blotting; (2) a subcellular localization of MT1-MMP in cellular protrusions (lamellipodia) and at the basal surface of migratory cells; (3) an inhibition of epithelial cell migration by BB94,

TIMP-2 and MT1-MMP antisense oligonucleotides; and (4) degraded fragments of Ln-5 $\gamma 2$ chain in migratory cultures of MCF10A cells.

Overexpression of MT1-MMP in epithelial cells associates with their migratory status

Using an in vitro migration assay, we have shown a stronger expression of MT1-MMP in cells located at the outgrowth periphery that, as determined by time lapse videomicroscopy, represents a subpopulation of migratory cells. In close relation to our observations obtained with the MCF10A cells, a downregulation of MT1-MMP associated with increased cell-cell contacts has been reported in a mouse mammary epithelial cell line (Tanaka et al., 1997). In vivo, MT1-MMP has also been reported in epithelial cells involved in processes that require cell migration, such as nephrogenesis (Tanney et al., 1998; Kanwar et al., 1999), placentation (Nawrocki et al., 1996; Bjorn et al., 1997; Tanaka et al., 1998) or tumour invasion (Sato and Seiki, 1996; Ellerbroek and Stack, 1999). Our in vitro model coupled to the videomicroscopy analyses nevertheless allowed us to demonstrate a specific induction of MT1-MMP both at the protein and mRNA level within the same cell line, in association with the expression of migratory properties. Furthermore, our results showing that BB94, TIMP-2 and MT1-MMP antisense oligonucleotides diminished MCF10A cell migration clearly emphasized a functional contribution of MT1-MMP overexpression and activity in MCF10A cell migration. It can therefore be suggested that MT1-MMP expression in epithelial cells can be temporally and spatially regulated during cell migration, and that it functionally contributes to this process.

Fig. 6: Ln-5 $\gamma 2$ chain degradation in migratory cultures of MCF10A cells and cleavage of Ln-5 $\gamma 2$ by MT1-MMP. (A) Western blot analyses of the Ln-5 $\gamma 2$ subunit in migratory cultures of MCF10A cells incubated in complete growth medium (MCF10A/FCS) 72 hours after the removal of the ring, in purified Ln-5 from SCC25 cells used as a control and in purified Ln-5 incubated with increasing amounts of the catalytic domain of human MT1-MMP (100 ng and 500 ng). (B) Western blot analyses of the Ln-5 $\gamma 2$ subunit 72 hours after the removal of the ring in migratory cultures of MCF10A cells incubated in complete growth medium (MCF10A/FCS), in EGF-induced migratory cultures (MCF10A/EGF) and in stationary cultures incubated in EGF/FCS-free medium (Control). (C) Western blot analyses of MT1-MMP in migratory cultures of MCF10A cells incubated in complete growth medium (MCF10A/FCS), in EGF-induced migratory cultures (MCF10A/EGF) and in stationary cultures incubated in EGF/FCS-free medium (Control). HT1080 cells are used as a control showing the three main forms of MT1-MMP at 63, 60 and 43 kDa.



MT1-MMP is distributed in lamellipodia and at the basal surface of migratory cells in contact with the substrate

More than demonstrating an increase of MT1-MMP expression in relation with cell migration, our results also showed a particular subcellular organization of MT1-MMP in migratory epithelial cells. Immunofluorescence analyses indeed clearly showed that MT1-MMP is mostly located in lamellipodia. Confocal microscopy confirmed these data but also revealed a punctiform labelling of MT1-MMP at the basal surface of migratory cells. Accordingly, Nakahara et al. have shown that RPMI-7951 melanoma cells transfected with MT1-MMP organized the protein in invadopodia at the basal surface of the cells and in lamellipodia at the leading edge of the cells, and displayed an enhanced ability to degrade FITC-labelled gelatin films (Nakahara et al., 1997). By contrast, they reported that ConA-induced overexpression of MT1-MMP, which did not generate the subcellular localisation in invadopodia, did not enhance gelatin degradation. It has also been shown that the degradation of the gelatin film was mostly accomplished by the enzyme present in the invadopodia rather than by the enzyme present in lamellipodia (Chen and Wang, 1999). Belien et al. (Belien et al., 1999) have also shown a distribution of MT1-MMP in the lamellipodia of MT1-MMP transfected rat glioma cells. Taken together with our results, these data suggest that the subcellular organization of MT1-MMP plays a major role in its degradative ability and emphasize the importance of a pericellular proteolysis in cell migration. Accordingly, Hotary et al. (Hotary et al., 2000) have shown that MT-MMPs, but not soluble MMPs, participate to cell invasion and morphogenesis of MDCK cells in collagen gels. A high level of expression of MT1-MMP and a subcellular organization at the leading edge of migratory cells and at their basal surface would thus contribute to a pericellular proteolysis involved in cell migration.

Laminin-5 deposition associates with MCF10A cell migration

We found that Ln-5, a potential substrate for MT1-MMP, is preferentially deposited by MT1-MMP-overexpressing migratory MCF10A cells at the periphery of the outgrowth. In agreement with our findings, several reports have shown that Ln-5 is expressed and deposited by migratory epithelial cells during wound healing both in vivo (Larjava et al., 1993; Kainulainen et al., 1998) and in vitro (Zhang and Kramer, 1996; Lotz et al., 1997; Qin and Kurpakus, 1998). Ln-5 has also been shown to be deposited adjacent to carcinoma cell clusters and has been related to the invasiveness of several types of tumours (Pyke et al., 1995; Sordat et al., 1998; Kosmehl et al., 1999; Maatta et al., 1999; Skyldberg et al., 1999; Lohi et al., 2000). Other evidence that the interaction between Ln-5 and MCF10A cells could be involved in their migration comes from our present results and those of Goldfinger et al. (Goldfinger et al., 1999) showing that the $\alpha 6$ and $\alpha 3$ integrin are not only present in MCF10A cells but also reorganized in relation with cell migration and that Ln-5 blocking antibodies decreased MCF10A cell migration. Accordingly, $\alpha 6\beta 4$ and $\alpha 3\beta 1$ are known as the main receptors for Ln-5 and have been implicated in adhesion but also in migration of epithelial cell lines on Ln-5 (Zhang and Kramer, 1996; Goldfinger et al., 1999). Furthermore, the overexpression and cellular redistribution of $\alpha 6\beta 4$ has been described in invasive tumours (Rabinovitz and Mercurio, 1996) and in wound healing (Kainulainen et al., 1998). It can thus be suggested that an increased deposition of Ln-5 by MCF10A cells at the periphery of the outgrowth and a reorganization of the Ln-5 integrin receptors in such cells are involved in their migratory properties.

Laminin-5 $\gamma 2$ chain degradation associates with MCF10A cell migration

Supporting the concept that MT1-MMP-mediated pericellular proteolysis could be involved in epithelial cell migration, we identified by western blotting increased amounts of fragments of the $\gamma 2$ chain in migratory cultures of MCF10A cells versus stationary cultures. The incubation of the recombinant catalytic domain of human MT1-MMP with purified human Ln-5 generated degraded fragments corresponding in size to those observed in our migratory cultures of MCF10A cells, suggesting a contribution of MT1-MMP in the generation of the Ln-5 $\gamma 2$ fragments associated with MCF10A cell migration. In apparent contrast to our data, Goldfinger et al. (Goldfinger et al., 1999) did not find the two smallest fragments of Ln-5 in the ECM of wounded MCF10A cell cultures that were allowed to heal for 8 hours. This discrepancy could be explained by the fact that, in our model, cells were allowed to migrate for 72 hours. This could indeed lead to an enrichment of the ECM with degraded Ln-5 $\gamma 2$ chain if, as suggested by our data, migratory cells newly synthesize Ln-5, overexpress MT1-MMP that subsequently cleaves the $\gamma 2$ chain of Ln-5. However, these authors found that plasmin-mediated modifications of the $\alpha 3$ subunit of Ln-5 regulated cell migration (Goldfinger et al., 1999). Taken together with our results, these data suggest that modifications of both $\gamma 2$ and $\alpha 3$ chains of Ln-5 can regulate MCF10A epithelial cell migration. Supporting our findings that Ln-5 $\gamma 2$ degradation associates with the overexpression of MT1-MMP and cell migration, the cleavage of the $\gamma 2$ chain of rat Ln-5 has been shown to be mediated by MMP-2 and/or MT1-MMP in a dose-dependent manner (Giannelli et al., 1997; Koshikawa et al., 2000), and not by other proteases such as plasmin or MMP-9 (Giannelli et al., 1997). The degraded fragments identified by Koshikawa et

al. (Koshikawa et al, 2000) after the cleavage of rat Ln-5 γ 2 chain by MT1-MMP differed in number and in size from those we observed after MT1-MMP-mediated degradation of human Ln-5 γ 2 chain. Also in agreement with our data, rat Ln-5 cleaved by MMP-2, but not intact rat Ln-5, has been reported to promote the migration of epithelial cells (Giannelli et al, 1997). It has also been shown using a transwell assay that human tumour cells constitutively expressing MT1-MMP display a higher migrating ability towards exogenously provided rat Ln-5 than MT1-MMP negative cells (Koshikawa et al, 2000). We report an enhanced production of both Ln-5 and MT1-MMP and a cleavage of Ln-5 γ 2 chain specifically associated with the expression of a migratory status by MCF10A epithelial cells.

In conclusion, our data demonstrate that the acquisition of a migratory phenotype by MCF10A epithelial cells is accompanied by an overexpression of MT1-MMP and a localization of the protein in the lamellipodia and at the basal surface of the cells in contact with the ECM substrate. This could participate to a pericellular degradation of the γ 2 chain of Ln-5, which is more specifically deposited by the migratory cells themselves, thereby providing a modified substrate that promotes cell migration.

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